Bioresource Technology 136 (2013) 257–266

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Combined alkali and acid pretreatment of spent mushroom substrate for reducing sugar and biofertilizer production

Hong-Ji Zhu [a], Jia-Heng Liu [a], Li-Fan Sun [b], Zong-Fu Hu [a], Jian-Jun Qiao [a,*]

[a] Key Laboratory of Systems Bioengineering, Ministry of Education, Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China
[b] School of Pharmacy, Tianjin Medical University, Tianjin 300070, China

HIGHLIGHTS

- An integrated process was built for utilization of spent mushroom substrate (SMS).
- Alkali pretreatment of SMS was investigated for the first time.
- Direct recycle of spent alkali liquor to reduce the cost of pretreatment.
- Combined alkali and acid pretreatment–enzymatic hydrolysis process was proposed.
- SMS residue from enzymatic hydrolysis was used for biofertilizer production.

ARTICLE INFO

Article history:
Received 20 December 2012
Received in revised form 25 February 2013
Accepted 27 February 2013
Available online 15 March 2013

Keywords:
Spent mushroom substrate
Lignocellulose
Pretreatment
Reducing sugar
Biofertilizer

ABSTRACT

Spent mushroom substrate (SMS) was pretreated with alkaline reagents including potassium hydroxide, lime and ammonia to enhance enzymatic saccharification. Under the best pretreatment conditions (1 M KOH, 80 °C, 90 min; 1 M lime, 80 °C, 120 min; 10 M ammonia, 70 °C, 120 min), the total reducing sugar (TRS) yield reached 258.6, 204.2 and 251.2 mg/g raw SMS, which were respectively 6.15, 4.86, and 5.98 times of untreated SMS. The effects of pretreatment by above alkaline reagents and sulfuric acid on the composition and structure of SMS were evaluated to provide comparative performance data. A new process, combined alkali and acid (CAA) pretreatment followed by enzymatic hydrolysis, was innovatively proposed to improve the cost-effectiveness and avoid environmental problems. The SMS residue after CAA pretreatment–enzymatic hydrolysis process was converted to biofertilizer with *Pichia farinose* FL7 and a cell density of 3.0×10^8 cfu/g in biomass was attained.

1. Introduction

The spent mushroom substrate (SMS) is a lignocellulosic byproduct of the mushroom industry. As estimated, there are about 2 million tons of SMS produced in China each year (Qiao et al., 2011), however, most of the SMS have been burnt for energy, which is neither environment-friendly nor economic. Furthermore, the lack of sustainable utilization strategies has greatly restricted the development of the mushroom industry (Finney et al., 2009). As a kind of lignocellulosic materials, SMS could be a source of reducing sugars for producing biofuels and other value-added biomaterials (White et al., 2008; Kaparaju et al., 2009). However, pretreatment by removing lignin and hemicellulose and breaking down the cellulose crystalline structure is required to overcome the problems caused by biomass recalcitrance for an effective cellulose conversion process. (Sun and Cheng, 2002; Himmel et al., 2007).

Sulfuric acid has been used to pretreat SMS by Qiao et al. (2011) and Kapu et al. (2012). The sulfuric acid pretreatment method involves high process temperature, which leads to high energy input and may also cause degradation of useful sugars and formation of fermentation inhibitors (Oliva et al., 2006; Panagiotou and Olsson, 2007). Compared to acid pretreatment strategy, alkali pretreatment generally proceeds under lower temperatures and pressures and its efficiency depends on the nature of the biomass feedstock, especially the lignin content (McMillan, 1994). Sodium hydroxide, ammonia and lime have been widely employed as alkaline pretreatment agents in lignocellulosic biomass and have been proved efficient at mild conditions (McIntosh and Vancov, 2010; Wu et al., 2011; Kim and Lee, 2005; Ko et al., 2009; Chang et al., 1997; Xu et al., 2010). In this study, we reported reducing sugar yields from SMS using three different alkaline reagents including potassium hydroxide (KOH), ammonia and lime under mild conditions for

* Corresponding author. Tel.: +86 022 2740 5836; fax: +86 022 8740 2107.
E-mail address: jianjunq@tju.edu.cn (J.-J. Qiao).

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.02.121

the first time. The effects of pretreatment by above alkaline reagents and sulfuric acid on the composition and structure of SMS were also evaluated to provide comparative performance data.

Costs and pollution are the main problems during the process of converting lignocellulose to reducing sugars. For conventional acid or alkali pretreatment–enzymatic hydrolysis process, the solid–liquid mixture has to be separated by filtration after pretreatment and then the solid fraction must be washed to remove the remaining alkali or acid. Furthermore, buffer is required for enzymatic hydrolysis to maintain a stable pH environment. All these procedures increase the cost of the pretreatment process. Moreover, the discharge of the spent pretreatment liquid will cause environmental problems such as water and soil contamination. In this research, in order to overcome these deficiencies, a cost-effective and zero-emission process, combined alkali and acid (CAA) pretreatment followed by enzymatic hydrolysis, was innovatively proposed to perform on SMS for reducing sugar production. In CAA pretreatment–enzymatic hydrolysis process, the resulting slurries after alkali and acid pretreatment respectively were mixed together to practice enzymatic hydrolysis. Therefore, this method without filtration, washing, buffer addition and spent liquid emission would have a good economic and environmental prospect.

Biofertilizers have been found to enhance crop yield and promote the sustainable development of agriculture industry. In our previous work, SMS was converted to biofertilizer using a stress-tolerant phosphate-solubilizing *Pichia farinose* FL7, which significantly improved the growth of soybean in pot experiments, demonstrating a tremendous potential in agricultural application (Zhu et al., 2012). However, 1.5% corn flour was added to provide nutritions during the biofertilizer preparation process, which increased production costs considerably. In this research, the SSF medium was prepared mainly from SMS residue after CAA pretreatment–enzymatic hydrolysis process, which was added as nutrients as well as a solid carrier for the growth of *P. farinose* FL7. The SMS hydrolysates containing reducing sugars served as a carbon source for *P. farinose* FL7 growth was added to the SSF medium. This design of SSF medium not only reduced the production costs greatly, but could supply phyto-essential elements remaining in SMS hydrolysates, such as ammonium, sulfate and potassium, which further enhanced the biofertilizer efficacy in promoting plant growth.

Therefore, the objectives of this research were to: (1) evaluate the TRS released from SMS with alkali pretreatment followed by enzymatic hydrolysis, (2) study the composition and structure change of SMS after alkali pretreatment, (3) investigate the efficacy of CAA pretreatment–enzymatic hydrolysis process and (4) determine the feasibility of cultivating *P. farinose* FL7 using SMS residue and hydrolysates after CAA pretreatment–enzymatic hydrolysis process for biofertilizer production. To the best of our knowledge, this is the first research reporting biofertilizer production using biomass residue from enzymatic hydrolysis.

2. Methods

2.1. Materials

SMS, the substrate after harvesting *Pleurotus ostreastus*, was obtained from Tianjin, PR China. The mushroom substrate used for planting *P. ostreastus* was composed of field hay, wheat straw, corn cobs and cotton seed hulls. SMS was ground to a particle size of 800 μm after drying and then stored in air-tight container at 4 °C for further use. Cellulase (NS50013) and xylanase (NS50014) preparations were kindly supplied by Novozymes A/S (Beijing, PR China). Enzyme activities as described by supplier are 70 FPU (Filter Paper Unit)/g and 600 XU (Xylanase Unit)/g respectively. All other chemicals used in this study were of analytical grade and purchased from Sigma Chemical Company (Shanghai, PR China).

2.2. Pretreatment

To evaluate the effect of pretreatment parameters (temperature, time, and alkali concentration) in alkali pretreatment, a $4 \times 4 \times 4$ factorial design was applied. KOH, ammonia and lime at temperatures of 50, 60, 70 and 80 °C in a static water bath were used to pretreat milled SMS samples at a solid loading of 10% (w/v) with pretreatment times of 30, 60, 90 and 120 min respectively. KOH and lime at concentrations of 0.25, 0.5, 0.75 and 1 M and ammonia at concentrations of 1, 5, 10 and 15 M were investigated. After alkali pretreatment, the samples were adjusted to room temperature and filtered through 0.2 μm nylon filter. Pretreated solids were washed with deionized water until the filtrate registered a neutral pH and dried at 105 °C for compositional analysis or enzymatic hydrolysis experiment.

The reuse of the spent KOH pretreatment liquid was conducted at 80 °C for 90 min with the solid loading of 10% (w/v). The filtrate was saved for pretreating the next batch of SMS at the same conditions. The reuse process was conducted four times.

The dilute sulfuric acid pretreatment of SMS was performed likewise except that an oil bath was employed. According to previous study (Qiao et al., 2011), the dilute sulfuric acid pretreatment was carried out under the optimal condition: temperature of 120 °C, concentration of 4%, pretreatment time of 120 min and solid to liquid rate of 1:16. All pretreatment experiments were carried out in triplicates.

2.3. Enzymatic hydrolysis

Three grams of alkali pretreated samples (dried at 105 °C for 6 h) were mixed with the 20 FPU cellulase and 200 XU xylanase per g solids, 60 ml of 50 mM acetate buffer (pH 4.8) was added, and the samples were incubated at 40 °C in a shaker bath for 72 h. After enzymatic hydrolysis, the samples were filtered through a filter paper and sugar analysis was performed on the supernatant.

The enzymatic hydrolysis of the dilute sulfuric acid pretreated SMS was performed under the same conditions. The CAA pretreatment–enzymatic hydrolysis process was detailed in Section 3.6. All enzymatic hydrolysis experiments were carried out in triplicates.

2.4. Analytical methods

2.4.1. Composition and sugar analysis

The lignin content of raw and pretreated SMS were determined according to National Renewable Energy Laboratory (NREL) standard methods Sluiter et al. (2008). Cellulose and hemicellulose content was determined by high performance liquid chromatography (HPLC) analysis using a Bio-Rad Aminex HPX-87P column and a refractive index detector (SHODEX). Sugars were analyzed at 65 °C using 0.00004% H_2SO_4 as the mobile phase (0.6 ml/min). Total reducing sugar (TRS) were determined using 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). All analyses were performed in triplicates.

2.4.2. Scanning electron microscopy (SEM) analysis

The structural differences in the lignocellulosic morphology of untreated and pretreated SMS were taken by scanning electron microscope (Hitachi S-4800, Tokyo, Japan). All images were taken at a magnification of 500×. The specimens to be coated were mounted on a conductive tape and coated with gold palladium using a JEOL-JFC-1200 fine coater and observed using a voltage of 10 kV.

2.4.3. X-ray diffraction (XRD) analysis

The dry samples untreated and pretreated SMS were pressed into disks for XRD analysis performed with a Bruker AXS system. The diffracted intensity of Cu K_α radiation ($\lambda = 0.1542$ nm; 40 kV and 30 mA) was measured in a 2θ range between 10° and 30°. The cellulose crystallinity index (CrI) was calculated using the empirical method of Segal et al. (1959):

$$\text{CrI}\ (\%) = [(I_{002} - I_{am})/I_{002}] \times 100$$

where I_{002} is the intensity of 002 peak at 2θ of 22.8°; I_{am} is the intensity attributed to amorphous portion at 2θ of 18°.

2.5. Biofertilizer preparation

With addition of SMS hydrolysates, solid residue from enzymatic hydrolysis were converted to biofertilizer using *P. farinose* FL7 by semi-solid fermentation (SSF). The SSF medium was prepared from dried SMS residue, hydrolysates and deionized water. 10, 20, 30, 40, 50 and 60 g hydrolysates which had been neutralized were added to 30 g dried SMS residue respectively in 250 ml Erlenmeyer flasks and deionized water was added to maintain an initial moisture content of 70%. Fermentation was performed by inoculating tested strain FL7 at about 1×10^6 cfu into the SSF media. The compost was turned over every 8 h for 10 days. Moisture content was determined by drying samples at 105 °C to a constant weight. Fungal growth was estimated by counting colony-forming units on agar plates. All the experiments were conducted in triplicates.

3. Results and discussion

3.1. Composition of SMS

The composition of SMS was related to the original ingredients of mushroom substrate (MS) and changes during mushroom cultivation process. Adopting the NREL protocol, SMS used in this study contained approximately 38.7% cellulose, 18.4% hemicelluloses and 20.2% lignin (Table 1) which were all lower than that in MS, especially hemicellulose. This might because hemicellulose is more susceptible to degradation than cellulose and lignin during the cultivation of *P. ostreastus*. Compared to other commonly used biomass for sugar production, such as corn stover and sorghum bagasse, SMS had higher lignin content (Table 1). Although this might lead to a more recalcitrant structure in SMS, its abundance and considerable disposal cost for the mushroom industry provided pressing needs to make effective use of SMS as a feedstock. Moreover, the large amount of holocellulose content (57.16%) showed its great potential to produce reducing sugars.

3.2. Effects of alkali pretreatment on TRS yield of SMS

Although alkali pretreatment could be performed at a wide range of conditions, moderate pretreatment severity (temperature: 50–80 °C; pretreatment time: 30–120 min) was selected in this study to improve the cost-effectiveness. The enzymatic hydrolysis of both cellulose and hemicellulose in pretreated SMS was critical for reducing sugar production. The TRS yield after enzymatic hydrolysis provided a measure of pretreatment effectiveness. Most other studies calculated the sugar production or conversion rate which was based on the amount of pretreated feedstock to evaluate pretreatment effect. Although they reported high sugar yield or conversion rate, they failed to take the cellulose and hemicellulose loss in pretreatment process into consideration. In this study, TRS yield resulted from per g of raw SMS was used which could reflect the comprehensive utilization rate of raw SMS. This section of work not only optimized the pretreatment condition for maximum TRS yield but examined the relationship between pretreatment conditions and enzymatic hydrolysis of pretreated SMS. Accoding to our previous study (Qiao et al., 2011), enzyme doses (20 FPU cellulase, 200 XU xylanase per g of solids) were applied to perform enzymatic hydrolysis at 40 °C for 72 h.

Elevation in temperature, alkali concentration and pretreatment time all increased TRS yield during KOH pretreatment (Fig. 1a). Temperature and KOH concentration had more significant impact than pretreatment time. An increase in temperature obviously improved TRS yield, which demonstrated that pretreatment at higher temperature was more acquiescent to enzymatic hydrolysis. While the pretreatment time and KOH concentration were fixed at 60 min and 1 M, the TRS yield was 209.2 mg/g and 244.3 mg/g raw SMS respectively at temperature of 50 and 70 °C, whereas the TRS yield showed little change as temperature increased from 70 to 80 °C, which indicated that further increase of temperature would not be conductive to releasing more TRS. Increase in concentration could improve TRS yield to varying degrees. Similar sugar yield in response to temperature and alkali concentration had been reported by McIntosh and Vancov (2010). Extending pretreatment time from 30 to 90 min obviously increased TRS yield. However, no apparent difference between the 90 and 120 min pretreatments was observed, which demonstrated pretreatment time of 90 min was sufficient for an effective KOH pretreatment process. Pretreating SMS with 1 M KOH for 90 min at 80 °C followed by enzymatic hydrolysis attained the highest TRS yield of 258.6 mg/g raw SMS, 6.15 times that of untreated SMS.

Lime was one of the most promising pretreatment reagents due to its proven effectiveness and low cost. As shown in Fig. 1b, increases in temperature, time and concentration all improved enzymatic saccharification of lime pretreated SMS. Pretreating SMS with 1 M lime for 120 min at 80 °C, followed by enzymatic hydrolysis, led to the highest TRS yield of 204.2 mg/g raw SMS, 4.86 times that of untreated SMS. However, with the same pretreatment time and concentration, only 118.5 mg/g raw SMS which declined by nearly half was attained at the low temperatures of 50 °C. An obvious rise of TRS yield with increasing pretreatment time at 50 °C was shown in Fig. 1b, which suggested that there were potentials of releasing more sugars under low temperature by extending pretreatment time. Xu et al. (2010) reported that the pretreatment effect of 50 °C, 24 h was comparable to that of 121 °C, 30 min using the same lime loading for switchgrass. In other researches of lime pretreatment (Chang et al., 1997; Xu et al., 2010), low lime loading of 0.1 g/g biomass was applied considering its poor solubility in water. Interestingly, although the lime usage in this study was beyond the amount required for maintaining the saturated lime solution, the increase of lime usage still improved TRS yield followed by enzymatic hydrolysis which proved the insoluble lime also took effect in an efficient pretreatment process. Taking the low price of lime into consideration, increasing lime usage to improve the pretreatment effect was practicable.

As shown in Fig. 1c, at all combinations of pretreatment time and temperature, TRS yield increased rapidly with increasing

Table 1
Composition of SMS.

Component	Composition (%)			MS[c]
	Corn stover[a]	Sorghum bagasse[b]	SMS[c]	
Cellulose	37.8	38.7	38.7	42.4
Hemicellulose	28.1	22.6	18.4	24.7
Lignin	17.8	15.4	20.2	22.4

[a] Reported by Yang and Wyman (2004).
[b] Reported by Wu et al. (2011).
[c] Data are averages of triplicates.

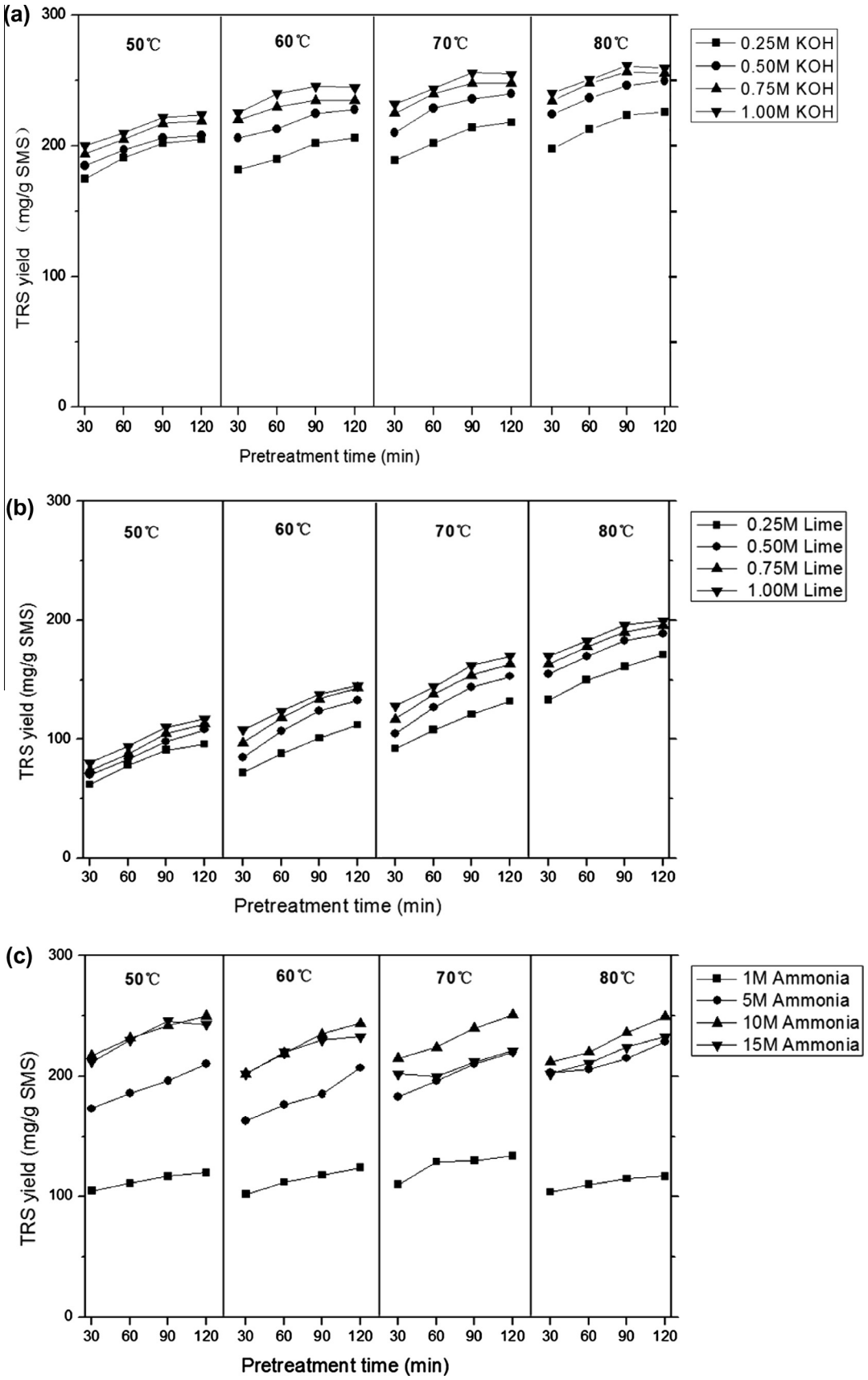

Fig. 1. TRS release from (a) KOH, (b) lime, (c) ammonia pretreated (10%, w/v) and enzyme hydrolyzed (40 °C, pH 4.8, 72 h) SMS presented as a function of alkali concentration, temperature and pretreatment time. The enzyme dose was 20 FPU cellulose and 200 XU xylanase per g solids. Sugar yields were expressed as mg/g raw SMS. Displayed data represented averages of three independent experiments.

ammonia concentration from 1 to 10 M. However, it reduced to varying degrees as the ammonia concentration increased from 10 to 15 M. It was worthwhile to note that TRS yield had no significant change as the temperature increased from 50 to 80 °C, which was different with KOH and lime pretreatment process. This suggested further increase of temperature wound not help promote enzymatic hydrolysis for ammonia pretreatment. TRS yield increased significantly as the pretreatment time extended from 30 to 120 min, which demonstrated pretreatment time also played an important role in the ammonia pretreatment process. This indicated that further studies needed to be done to determine whether the TRS yield could get a significant improvement with a longer pretreatment time and the increased TRS yield could potentially compromise the cost-effectiveness of shorter pretreatment time. Pretreatment with 10 M ammonia for 120 min at 70 °C resulted in a maximum TRS yield of 251.2 mg/g raw SMS which was 5.98 times that of untreated SMS. However, pretreatment at 50 °C with the same ammonia concentration and time led to 249.2 mg/g raw SMS, only 2.0 mg/g lower than that that at 70 °C. Thus, from the point of view of energy-saving, pretreatment with 10 M ammonia concentration for 120 min at 50 °C could be used in industrial application.

As shown in Table 2, the reducing sugars obtained by enzymatic hydrolysis mainly consisted of glucose, xylose and a small amount of arabinose. Hydrolysis of KOH, lime, ammonia pretreated samples resulted in a significant increase of glucose yield which was 9.2, 8.1 and 9.8 times that of untreated SMS, respectively. This indicated that the increased TRS yield after alkali pretreatments was mainly attributed to the improvement of cellulose conversion. The result in Table 2 suggested alkali pretreatment had a limited impact on hemicellulose digestibility with a highest xylose yield of 53.7 mg/g raw SMS from KOH pretreatment, 2.9 times that of untreated SMS. Other monosaccharide sugars derived from hemicellulose, such as galactose and mannose, were also measured but not reported due to their low concentrations in hydrolysates.

3.3. Effects of alkali pretreatment on the composition and structure of SMS

KOH, lime and ammonia pretreated samples under optimal pretreatment conditions which led to maximum TRS yield respectively were chosen for analyzing the composition and structure change. Sulfuric acid pretreated SMS sample was also investigated for comparison.

The pretreatment results expressed as solid loss and removal of three main chemical components (cellulose, hemicellulose and lignin) in each pretreatment were listed in Table 3. Sulfuric acid pretreatment caused the greatest solid loss of 42.4% followed by KOH (37.5%) and then ammonia (34.2%). The lowest reduction in solids (only 25.6%) was observed after lime pretreatment probably because lime acted as a weak base due to its poor solubility in water. The solid loss was mainly caused by the solubilization of components such as lignin, hemicellulose and other soluble extractives in SMS. Alkali (KOH, lime and ammonia) pretreatments caused only minor cellulose removal; higher than 92% cellulose of raw SMS was recovered which indicated that under moderate conditions alkali pretreatment resulted in little cellulose loss. The removal of hemicellulose and lignin were greatly different. KOH and lime pretreatment resulted in slight hemicellulose removal of 6.7% and 3.2% respectively, whereas ammonia 33.3%. KOH and ammonia pretreatment significantly removed the lignin content (69.5% and 52.4%, respectively) followed by lime (31.2%). An apparent correlation between lignin removal and TRS yield of alkali pretreated SMS samples was observed. More detailed investigation was carried out to examine whether lignin removal played a crucial role in enhancing enzymatic saccharification of alkali pretreated SMS in Section 3.4. In contrast, pretreatment with sulfuric acid had minor impact on the amount of lignin and cellulose but effectively removed 84.7% of hemicellulose which indicated sulfuric acid mainly hydrolysed the hemicellulose fraction of lignocellulosic biomass. The improvement in enzymatic hydrolysis of sulfuric acid pretreated SMS over untreated feedstock (Kapu et al., 2012) could be largely attributed to the removal of hemicellulose. Similar conclusions were previously reported by Kabel et al. (2007) for wheat straw and Kumar et al. (2009) for corn stover.

SEM images of untreated and pretreated SMS samples were shown in Supplementary Fig. S1. The untreated SMS sample displayed a flat, compact and smooth surface, indicating a highly regular structure, while all the alkali and acid pretreated samples showed a loose and disordered structure and missed some parts of the outer surface compared with the images for untreated samples. The SEM images showed how the structure of native SMS changed after the pretreatment, which increased the surface area for enzymatic hydrolysis. Identical observations were earlier reported by Ko et al. (2009) for rice straw with ammonia and Sindhu et al. (2011) for sugarcane tops with dilute sulfuric acid.

Crystallinity was believed to be an important feature affecting enzymatic hydrolysis of lignocellulosic biomass (Fan et al., 1980). Reducing the crystallinity of the cellulose considerably improved the enzymatic hydrolysis efficiency of pure cellulose (Hall et al., 2010). However, when it comes to lignocellulosic biomass, the relationship between cellulose crystallinity and enzymatic hydrolysis was more complicated due to its more complex components and structure. XRD profile of untreated, alkali pretreated and sulfuric acid pretreated SMS were shown in Supplementary Fig. S2. Two typical diffraction peaks were observed at $2\theta = 15.0°$ and 22.8° for all the samples, which corresponded to (101) and (002) lattice planes of crystalline cellulose I. Compared to raw SMS, the peak (101) and peak (002) of alkali pretreated samples all became weak and narrow, while the acid pretreated sample presented a similar peak (101) and broader peak (002). The CrI values were determined based on the XRD patterns and depicted in Table 3. The

Table 2
Sugar analysis in enzymatic hydrolysates of pretreated SMS.

Pretreatment[a]	Sugar yield (mg/g raw SMS)			
	Glucose	Xylose	Arabinose	TRS
Untreated	22.1	18.5	1.5	42.1
KOH	203.4	53.7	2.3	258.6
Lime	179.4	22.3	1.6	204.2
Ammonia	215.6	32.1	2.5	251.2

All data are averages of triplicates.
[a] Pretreatment conditions: 1 M KOH, 80 °C, 90 min; 1 M lime, 80 °C, 120 min; 10 M ammonia, 70 °C, 120 min.

Table 3
Solid loss, removal of three main components (cellulose, hemicellulose and lignin) and CrI of SMS samples.

Pretreatment[a]	Solid loss[b] (%)	Removal rate[b] (%)			CrI (%)
		Cellulose	Hemicellulose	Lignin	
Untreated	–	–	–	–	37.8
KOH	37.5	4.4	6.7	69.5	58.4
Lime	25.6	1.6	3.2	31.2	47.5
Ammonia	34.2	7.3	33.3	54.2	32.6
Sulphuric acid	42.4	7.6	84.7	8.5	59.6

All data are averages of triplicates.
[a] Pretreatment conditions: 1 M KOH, 80 °C, 90 min; 1 M lime, 80 °C, 120 min; 10 M ammonia, 70 °C, 120 min; 4% (w/w) sulphuric acid, 120 °C, 120 min.
[b] Values based on the initial dried weight of SMS.

CrI of untreated ample was 37.8% due to the high content of amorphous substances such as lignin and hemicellulose present in the sample. The CrI of KOH and lime pretreated SMS was raised to 58.4% and 47.5%. The increase was mainly caused by the removal of amorphous components such as lignin which increased the relative amount of crystalline matter. The result also suggested that during the moderate conditions in KOH and lime pretreatment process, there were little destruction to the crystal structure of cellulose. The slight CrI increase of lime pretreatment was earlier reported by Chang and Holtzapple (2000). Ammonia pretreatment caused some decline of CrI, which indicated that under high concentration of ammonia (10 M), the crystalline structure of cellulose was disrupted. Wu et al. (2011) reported that the CrI of alkali pretreated bagasse showed a tendency to decline with increasing alkali concentration. After sulfuric acid pretreatment, the CrI of SMS sample increased to 59.6%, which was probably caused by the significant removal of hemicellulose. Similar CrI increase of acid pretreated samples has been reported by Sindhu et al. (2010, 2011) for sugarcane bagasse and sugarcane tops.

3.4. Effect of lignin and hemicellulose removal on SMS enzymatic hydrolysis improvement

Lignin was one of the major barriers for enzymatic hydrolysis (Chang and Holtzapple, 2000). The limitation to enzymatic hydrolysis was typically from three aspects: blocking the accessibility of enzyme, adsorbing the enzyme and forming lignin–carbohydrate complex. Thus, the removal of lignin could efficiently improve enzymatic hydrolysis (Yang and Wyman, 2004; Yu et al., 2011). Besides, the removal of hemicellulose had also been reported to enhance enzymatic saccharification (Mussatto et al., 2008).

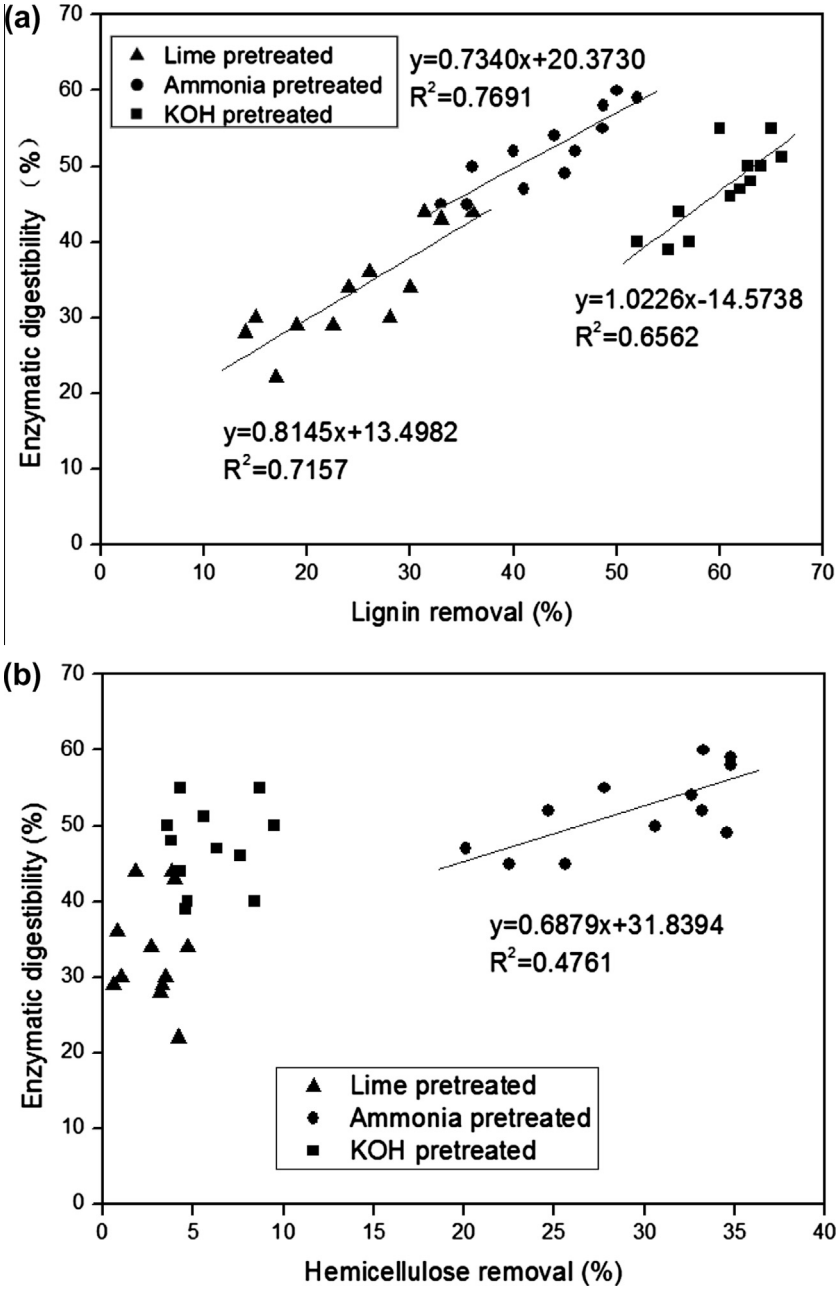

Fig. 2. Correlation between lignin (a) and hemicellulose; (b) removal and enzymatic digestibility of alkali pretreated SMS.

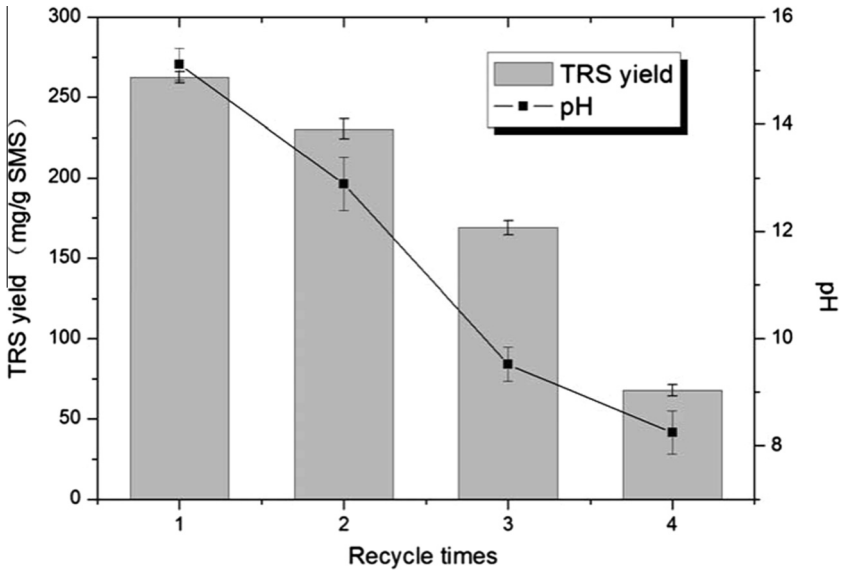

Fig. 3. Effect of direct recycle of KOH solution on pH value and TRS yield. pH value was measured before pretreatment. Recycle time 1 indicated that the KOH solution was fresh (concentration of 1 M). Recycle time 2 indicated the KOH solution had been used for 1 batch pretreatment and the rest was deduced by analogy.

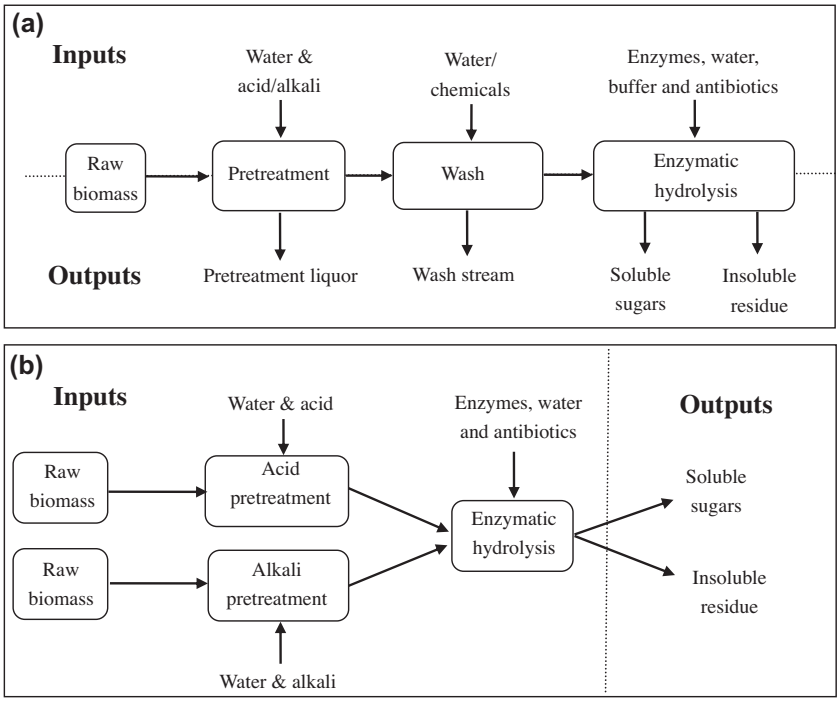

Fig. 4. Input–output diagrams of (a) conventional acid or alkali pretreatment–enzymatic hydrolysis process and (b) CAA pretreatment–enzymatic hydrolysis process.

Within each alkali pretreatment trial, 12 pretreated samples were randomly selected to examine the relationship between lignin and hemicellulose removal and enzymatic digestibility of alkali pretreated SMS, regardless of specific pretreatment conditions (alkali concentration, pretreatment time and temperature). Based on the theoretical maximum yield of glucose and xylose, enzymatic digestibility was defined as follows:

$$\text{Enzymatic digestibility} = \frac{(0.9 \times \text{glucose} + 0.88 \times \text{xylose}) \text{ in enzymatic hydrolysates}}{(\text{glucan} + \text{xylan}) \text{ in pretreated SMS}} \times 100\%$$

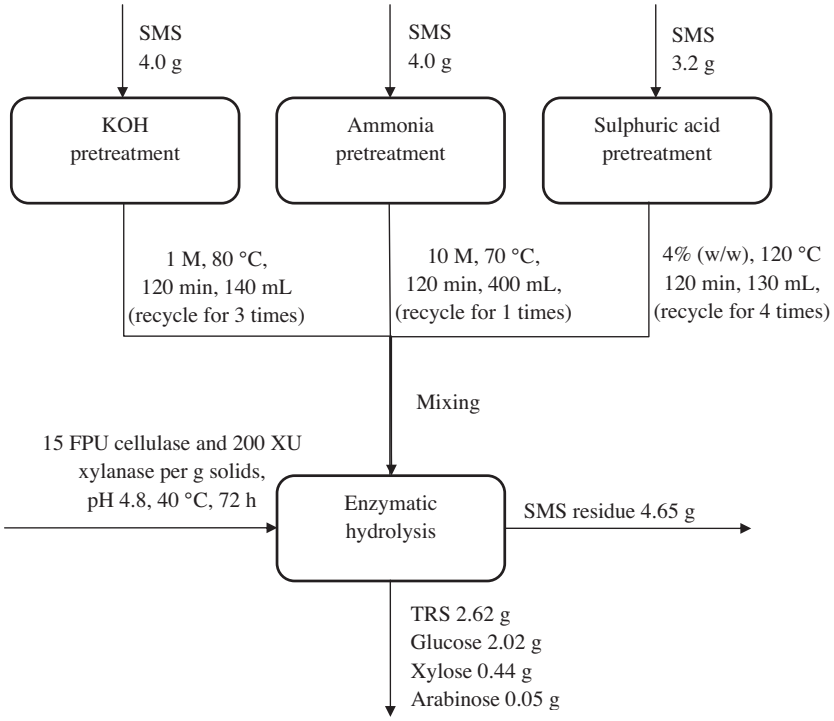

Fig. 5. Mass balance of the overall CAA pretreatment–enzymatic hydrolysis process.

The enzymatic digestibility of alkali pretreated SMS as a function of lignin removal was presented in Fig. 2a. Pretreatments with the three alkaline reagents (KOH, lime and ammonia) all obtained an obverse correlation between the two variables with a correlation coefficient of 0.6562, 0.7157 and 0.7691, respectively, which indicated that the efficiency of enzymatic hydrolysis obviously increased with the rise of lignin removal. These correlation coefficients were close to that of other researches such as sodium hydroxide pretreatment of sorghum bagasse (Wu et al., 2011), lime pretreatment of corn stover (Kim and Holtzapple, 2006) and ammonia pretreatment of rice straw (Jung et al., 2011). The slight difference was probably owing to the different physical structures and chemical natures of feedstocks. Compared with KOH pretreatment, ammonia pretreated SMS showed comparable enzymatic digestibility with lower lignin removal. This further confirmed the removal of hemicellulose and decline of cellulose crystallinity could enhance enzymatic saccharification significantly. It was important to note that enzymatic digestibility increased more sharply under high delignification conditions in KOH pretreatment, which suggested that further studies on delignification were critical to achieving ideal enzymatic digestibility.

The effect of hemicellulose removal on enzymatic digestibility was also investigated. As shown in Fig. 2b, for ammonia pretreatment, hemicellulose removal ranged from 20.1% to 34.8%. As the removal of hemicellulose increased, enzymatic digestibility increased proportionally with a correlation coefficient of 0.4761. This indicated the removal of hemicellulose was also beneficial for improving enzymatic saccharification of SMS but the relevance was smaller compared with lignin. However, no obvious relationship between hemicellulose removal and enzymatic digestibility was found in KOH and lime pretreatment probably due to their low hemicellulose removal.

3.5. Direct recycle of spent alkali liquor

An efficient method to reduce the pretreatment cost was direct recycle of the pretreatment liquor. As lime pretreatment resulted in a relatively low TRS yield and the separation of insoluble lime was difficult, only the recycle of KOH and ammonia solution were investigated. The data in Fig. 3 suggested that TRS yield after enzymatic hydrolysis declined with recycle times of KOH solution. Meanwhile, the pH of obtained spent liquor showed distinct decrease mainly due to the alkalinity consumption by the biomass such as saponification reaction between alkali and lignin or other components in pretreatment process (Sun and Cheng, 2002). Pedersen and Meyer (2010) had reported that at mild conditions the pretreatment pH was a significant factor influencing the removal of lignin and the subsequent enzymatic hydrolysis. The results of present study were in accordance with their conclusion. Besides, the solubility of lignin in spent liquor might also play a dominant role in recycling process (Zhao and Liu, 2012). The pretreatment effect of KOH solution after recycle for 3 times was poor (Fig. 3) which probably because the dissolved lignin concentration in the spent liquor reached to saturated state. According to Fig. 3, the KOH solution could be recycled for 3 times. This meant 2/3 of KOH consumption was saved, which would make great sense to decrease the cost of pretreatment. However, the spent ammonia liquor showed low efficiency for recycle (data not shown) presumably due to its highly volatile ability. Qiao et al. (2011) had reported the sulfuric acid solution could be recycled to process fresh SMS for four times.

3.6. CAA pretreatment–enzymatic hydrolysis process

Costs and pollution were the main drawbacks for converting lignocellulose to reducing sugars. As shown in Fig. 4a, in conventional acid or alkali pretreatment–enzymatic hydrolysis process, the emission of spent alkali or acid pretreatment liquid caused environmental pollution and chemical wastes. Moreover, the buffer added for enzymatic hydrolysis also increased the cost of the whole process. Besides, some reducing sugars were attained in acid pretreatment liquor (Qiao et al., 2011), the separation and utilization of these sugars made the process more complex. The method of CAA pretreatment followed by enzymatic hydrolysis

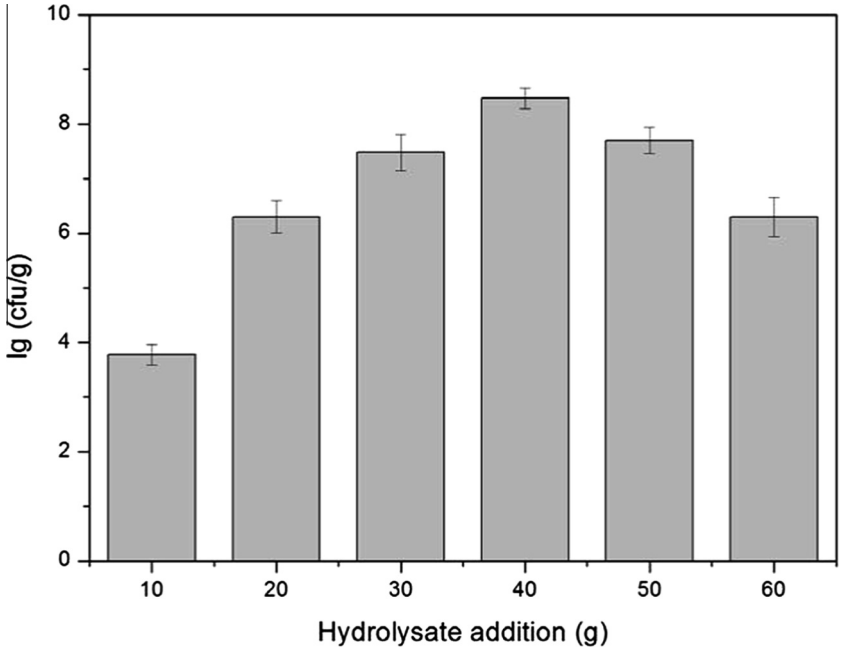

Fig. 6. Cell density of *P. farinose* FL7 grown in SMS residue with addition of SMS hydrolysates. Displayed data represented averages of three independent experiments.

was innovatively proposed in this study to realize a low-cost and zero-emission process (Fig. 4b). KOH and ammonia were used as alkaline reagents and sulfuric acid as acid reagent for their proven pretreatment efficiency.

An overall mass balance diagram describing the process from CAA pretreatment to enzymatic hydrolysis was shown in Fig. 5. For CAA pretreatment, 4.0 g SMS was soaked in a 1 M KOH solution (3 times recycle) at a solid loading of 10% and pretreated for 90 min at 80 °C. Another 4.0 g SMS was soaked in a 10 M ammonia solution at a solid loading of 10% and pretreated for 120 min at 70 °C. 3.2 g SMS was soaked in a 4% sulphuric acid solution (4 times recycle) at a solid to liquid ratio of 1:16 and pretreated for 120 min at 120 °C. Then the three batches of solid–liquid mixtures were mixed together for enzymatic hydrolysis. Enzymes were directly added to the obtained mixture with a loading of 20 FPU cellulase and 200 XU xylanase per g solids, and then enzymatic hydrolysis was performed at 40 °C for 72 h. After enzymatic hydrolysis, 4.65 g solids were recovered which could be converted directly to biofertilizer as discussed in Section 3.7. Meanwhile, 2.62 g TRS containing 2.02 g glucose, 0.46 g. xylose, and 0.05 g arabinose were attained in the enzymatic hydrolysates. These values are important in determining the overall yield of ethanol or other bioproducts in fermentation which is the next processing step following enzymatic hydrolysis.

The mass balance of CAA pretreatment–enzymatic hydrolysis process indicated that a TRS yield of 233.6 mg/g raw SMS was attained. The inhibitors such as lignin degradation products might cause some loss of enzyme activity. Increased enzyme dosage of 25 FPU cellulase and 250 XU xylanase per g solids led to a TRS yield of 254.7 mg/g raw SMS which was comparable to that of KOH pretreatment followed by enzymatic hydrolysis with 20 FPU cellulase and 200 XU xylanase per g solids. This demonstrated a higher enzyme loading was required in enzyme hydrolysis after CAA pretreatment in order to compensate for the loss of free enzyme activity. However, it should be noticed that CAA pretreatment–enzymatic hydrolysis process was conducted without filtration, washing, buffer addition and emission of pretreatment liquor. Furthermore, sugars produced after sulphuric acid pretreatment and that after enzymatic hydrolysis were both existed in the final hydrolysates which could be used for fermentation conveniently. Therefore, an economic evaluation considering the total process was needed for further comparing the CAA pretreatment–enzymatic hydrolysis process with conventional pretreatment–enzymatic hydrolysis processes.

3.7. Biofertilizer preparation

The SSF medium was mainly prepared from dried SMS residue and hydrolysates after CAA pretreatment–enzymatic hydrolysis process. SMS residue was added as nutrients for the growth of *P. farinose* FL7 as well as a solid carrier. The SMS hydrolysates containing reducing sugars could serve as a carbon source for microbes (Qiao et al., 2011).

The SSF medium facilitated the growth and biomass production of *P. farinose* FL7 during the fermentation significantly. A rapid increase in biomass concentration within the initial 5 days was observed and the growth slowed down during the later stages of fermentation probably due to limitation of nutrients. The best addition of SMS hydrolysates was 40 g which led to the maximum colony density of 3.0×10^8 cfu/g biofertilizer (Fig. 6) at the fifth day which illustrated 40 g hydrolysates contained sufficient sugars for the growth of *P. farinose* FL7. However, the addition of more than 40 g hydrolysates resulted in a lower biomass accumulation probably due to the inhibition effect high concentration of ions, such as ammonium, sulfate and potassium exerted on the growth of *P. farinose* FL7. This indicated the SMS residue and a small portion (about 9%) of obtained hydrolysates could be used for producding biofertilizer through a SSF process.At the end of fermentation, the SSF medium became softer and darker due to the degradation of SMS residue by the inocula. Moisture of the medium reduced to about 60% at the end of fermentation from the initial value of 70%. A decrease of pH from 7.0 to 5.2 was also observed.

In other researches of conversing agricultural wastes to biofertilizer, nutritions especially carbon source, such as starch (Ogbo, 2010) and corn flour (Zhu et al., 2012), were added for the growth of biofertilizer organism during fermentation. In this study, the SMS hydrolysates were added to supply nutritions in the biofertilizer production, which provided a novel application of

the hydrolysates. Such a product would not only present a viable means of disposing treated biomass wastes, but could supply phyto-essential elements remaining in SMS hydrolysates, such as ammonium, sulfate and potassium which further enhanced the biofertilizer efficacy, which could result in considerable economic and environmental benefits.

4. Conclusions

The study showed that SMS could be a potential feedstock for reducing sugar production by alkali pretreatment under moderate conditions. A positive correlation between lignin removal and enzymatic digestibility was observed. The hemicellulose removal and cellulose crystallity decrease could also enhance enzymatic saccharification. As a low-cost and zero-emission process, CAA pretreatment followed by enzymatic hydrolysis proved effective and further improvement was possible if the inhibitors in the solution were removed. In summary, converting SMS to reducing sugars and producing biofertilizer with SMS residue from hydrolysates represented a valuable pathway for comprehensive utilization of a mushroom industry by-product.

Acknowledgements

This research is supported by the Key Technologies R&D Program of Tianjin (Grant No. 12ZCZDNC01700). We are grateful to the State Key Laboratory of Chemical Engineering of Tianjin University for providing equipment and facilities.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.02.121.

References

Chang, V.S., Holtzapple, M.T., 2000. Fundamental factors affecting biomass enzymatic reactivity. Appl. Biochem. Biotechnol. 84, 5–37.
Chang, V.S., Burr, B., Holtzapple, M.T., 1997. Lime pretreatment of switchgrass. Appl. Biochem. Biotechnol. 63, 3–19.
Fan, L.T., Lee, Y.H., Beardmore, D.H., 1980. Mechanism of the enzymatic hydrolysis of cellulose: effect of major structural features of cellulose on enzymatic hydrolysis. Biotechnol. Bioeng. 23, 177–199.
Finney, K.N., Ryu, C., Sharifi, V.N., Swithenbank, J., 2009. The reuse of spent mushroom compost and coal tailings for energy recovery: comparison of thermal treatment technologies. Bioresour. Technol. 100, 310–315.
Hall, M., Bansal, P., Lee, J.H., Realff, M.J., Bommarius, A.S., 2010. Cellulose crystallinity – a key predictor of the enzymatic hydrolysis rate. FEBS J. 277, 1571–1582.
Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D., 2007. Biomass recalcitrance. engineering plants and enzymes for biofuels production. Science 315, 804–807.
Jung, Y.H., Kim, I.J., Kim, J.J., Oh, K.K., Han, J.I., Choi, I.G., Kim, K.H., 2011. Ethanol production from oil palm trunks treated with aqueous ammonia and cellulase. Bioresour. Technol. 102, 7307–7312.
Kabel, M.A., Bos, G., Zeevalking, J., Voragen, A.G.J., Schols, H.A., 2007. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. Bioresour. Technol. 98, 2034–2042.
Kaparaju, P., Serrano, M., Thomsen, A.B., Kongjan, P., Angelidaki, I., 2009. Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresour. Technol. 100, 2562–2568.
Kapu, N.U.S., Manning, M., Hurley, T.B., Voigt, J., Cosgrove, D.J., Romaine, C.P., 2012. Surfactant-assisted pretreatment and enzymatic hydrolysis of spent mushroom compost for the production of sugars. Bioresour. Technol. 114, 399–405.
Kim, S., Holtzapple, M.T., 2006. Effect of structural features on enzyme digestibility of corn stover. Bioresour. Technol. 97, 583–591.
Kim, T.H., Lee, Y.Y., 2005. Pretreatment of corn stover by soaking in aqueous ammonia. Appl. Biochem. Biotechnol. 121, 1119–1131.
Ko, J.K., Bak, J.S., Jung, M.W., Lee, H.J., Choi, I.G., Kim, T.H., Kim, K.H., 2009. Ethanol production from rice straw using optimized aqueous-ammonia soaking pretreatment and simultaneous saccharification and fermentation processes. Bioresour. Technol. 100, 4374–4380.
Kumar, R., Mago, G., Balan, V., Wyman, C.E., 2009. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. Bioresour. Technol. 100, 3948–3962.
McIntosh, S., Vancov, T., 2010. Enhanced enzyme saccharification of Sorghum bicolor straw using dilute alkali pretreatment. Bioresour. Technol. 101, 6718–6727.
McMillan, J., 1994. Enzymatic conversion of biomass for fuels production. In: Himmel, M., Baker, J., Overend, R. (Eds.), Pretreatment of Lignocellulosic Biomass. American Chemical Society, Washington, DC, pp. 292–324.
Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.
Mussatto, S.I., Fernandes, M., Milagres, A.M.F., Roberto, I.C., 2008. Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain. Enzyme. Microb. Technol. 43, 124–129.
Ogbo, F.C., 2010. Conversion of cassava wastes for biofertilizer production using phosphate solubilizing fungi. Bioresour. Technol. 101, 4120–4124.
Oliva, J.M., Negro, M.J., Sáez, F., Ballesteros, I., Manzanares, P., González, A., Ballesteros, M., 2006. Effects of acetic acid, furfural and catechol combinations on ethanol fermentation of *Kluyveromyces marxianus*. Process Biochem. 41, 1223–1228.
Panagiotou, G., Olsson, L., 2007. Effect of compounds released during pretreatment of wheat straw on microbial growth and enzymatic hydrolysis rates. Biotechnol. Bioeng. 96, 250–258.
Pedersen, M., Meyer, A.S., 2010. Lignocellulose pretreatment severity – relating pH to biomatrix opening. New Biotechnol. 27, 739–750.
Qiao, J.J., Zhang, Y.F., Sun, L.F., Liu, W.W., Zhu, H.J., Zhang, Z.J., 2011. Production of spent mushroom substrate hydrolysates useful for cultivation of Lactococcus lactis by dilute sulfuric acid, cellulase and xylanase treatment. Bioresour. Technol. 102, 8046–8051.
Segal, L., Creely, J.J., Martin Jr., A.E., Conrad, C.M., 1959. An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. Tex. Res. J. 29, 786–794.
Sindhu, R., Binod, P., Satyanagalakshmi, P., Janu, K.U., Sajna, K.V., Kurien, N., Sukumaran, R.K., Pandey, A., 2010. Formic acid as a potential pretreatment agent for the conversion of sugarcane bagasse to bioethanol. Appl. Biochem. Biotechnol. 162, 2313–2323.
Sindhu, R., Kuttiraja, M., Binod, P., Janu, K.U., Sukumaran, R.K., Pandey, A., 2011. Dilute acid pretreatment and enzymatic saccharification of sugarcane tops for bioethanol production. Bioresour. Technol. 102, 10915–10921.
Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Determination of structural carbohydrates and lignin in biomass. National Renewable Energy Laboratory Analytical Procedure (LAP). NREL/TP-510-42618, Golden, CO.
Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol. 83, 1–11.
White, J.S., Yohannan, B.K., Walker, G.M., 2008. Bioconversion of brewer's spent grains to bioethanol. FEMS Yeast Res. 8, 1175–1184.
Wu, L., Arakane, M., Ike, M., Wada, M., Takai, T., Gau, M., 2011. Low temperature alkali pretreatment for improving enzymatic digestibility of sweet sorghum bagasse for ethanol production. Bioresour. Technol. 102, 4793–4799.
Xu, J., Cheng, J.J., Sharma-Shivappa, R.R., Burns, J.C., 2010. Lime pretreatment of switchgrass at mild temperatures for ethanol production. Bioresour. Technol. 101, 2900–2903.
Yang, B., Wyman, C.E., 2004. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. Biotechnol. Bioeng. 86, 88–98.
Yu, Z.Y., Jameel, H., Chang, H.M., Park, S., 2011. The effect of delignification of forest biomass on enzymatic hydrolysis. Bioresour. Technol. 102, 9083–9089.
Zhao, H.B., Liu, D.H., 2012. Fractionating pretreatment of sugarcane bagasse by aqueous formic acid with direct recycle of spent liquor to increase cellulose digestibility – the Formiline process. Bioresour. Technol. 117, 25–32.
Zhu, H.J., Sun, L.F., Zhang, Y.F., Zhang, X.L., Qiao, J.J., 2012. Conversion of spent mushroom substrate to biofertilizer using a stress-tolerant phosphate-solubilizing *Pichia farinose* FL7. Bioresour. Technol. 111, 410–416.